

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/28, 1/54, G01N 27/327	A1	(11) International Publication Number: WO 97/22715 (43) International Publication Date: 26 June 1997 (26.06.97)
(21) International Application Number: PCT/US96/18889 (22) International Filing Date: 25 November 1996 (25.11.96) (30) Priority Data: 08/563,728 18 December 1995 (18.12.95) US (71) Applicant: ABBOTT LABORATORIES [US/US]; Chad 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US). (72) Inventors: HENNING, Timothy, P.; 189 Sheffield Lane, Vernon Hills, IL 60061 (US). SPRING, Thomas, G.; 234 Barberry, Highland Park, IL 60035 (US). (74) Agents: WEINSTEIN, David, L. et al.; Abbott Laboratories, CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: INTERFERENCE FREE BIOSENSOR (57) Abstract Provided are microparticle forms of carbon, carbon catalysts and carbon-containing electrically conductive materials which are covalently linked to peroxidase. The carbon:peroxidase conjugates are suitable for use as substrates in conventional electrodes. Surprisingly, the conjugates display very little sensitivity to known interfering substances and thus are suitable for use as interference free electrodes.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INTERFERENCE FREE BIOSENSOR

Field of the Invention

5 The present disclosure is in the field of biosensors for the analysis of body fluids.

Background of the Invention

One method for measuring the concentration of glucose in blood is to cause a reaction between the glucose and the enzyme glucose oxidase.
10 The reaction produces hydrogen peroxide, which can be electrochemically oxidized at positive potentials. The current produced by the oxidation of peroxide is measured and is proportional to the glucose concentration in the blood. A similar scheme may be used to measure other analytes of interest by selecting the appropriate enzyme that will produce hydrogen
15 peroxide in proportion to the concentration of the analyte, e.g., lactate may be similarly measured using the enzyme lactate oxidase.

A long standing problem with these measurement techniques is that of interference from other substances. Blood may contain other components which can oxidize at the same potential as hydrogen
20 peroxide; prevalent among these are urate and acetaminophen. As these or other interfering substances oxidize, the current resulting from their oxidation is added to and indistinguishable from the current resulting from the oxidation of the hydrogen peroxide. An error therefore results in the quantification of the peroxide and consequently the blood analyte.

25 Many methods have been tried to reduce or eliminate these interferences. Acetaminophen has been particularly difficult to eliminate because, unlike urate, acetaminophen is not charged. One methodology disclosed in the art (see, e.g., Sittampalam, G., and Wilson, G. S., Analytical Chemistry, 55:1608-1610, 1983, and Geise, R. J., et al, Biosensors and
30 Bioelectronics, 6:151-160, 1991) has been to use one or more selectively permeable membranes to prevent the interfering substances from contacting the electrode, although this method is not effective at fully preventing interference.

An alternate method for preventing interference is disclosed in U.S.
35 Patents 5,225,064 and 5,334,296. This method decreases the electrical

- 2 -

potential of the reaction to a state where urate and acetaminophen are not oxidized. This may be accomplished by employing an enzyme that will reduce hydrogen peroxide, such as a form of peroxidase, in the electrode. In this type of system, the hydrogen peroxide reacts with the peroxidase, in which reaction the hydrogen peroxide is reduced and the peroxidase oxidizes. If the peroxidase is in intimate contact with an electrode's surface, the peroxidase can be reduced at the electrode by imposing an electrical potential. The magnitude of the current required to reduce the peroxidase is proportional to the concentration of the hydrogen peroxide and hence proportional to the concentration of glucose or other analyte of interest. Moreover, the reactions occur at potentials at which urate and acetaminophen do not oxidize and therefore do not interfere.

The art recognizes the use of horseradish peroxidase or fungal peroxidase physically adsorbed to a gold or carbon surface in attempts to prevent interference (see, e.g., U.S. Patents 5,225,064 and 5,334,296; Ho, W. O., et al, J. Electroanal. Chem., 351:187-197, 1993; Wollenberger, U., et al, Bioelectrochemistry and Bioenergetics, 26:287-296, 1991; Csoregi, E., et al, Anal. Chem. 66:3604-3610, 1994). However these reports indicate that such use has yielded unsatisfactory results as the magnitude of the electric currents forming the signals in these applications are quite small and the linearity is poor at high peroxide concentrations.

There is great utility, therefore, in the development of an electrode system in which hydrogen peroxide reacts in a measurable way, generating signal currents of usable magnitudes and that are linear to high concentrations, yet which are not subject to interference caused by the oxidation of interfering substances such as urate and acetaminophen.

Brief Description of the Figures

Figure 1 shows an analytical electrode embodying the interference free biosensor of the invention.

Summary of the Invention

The present disclosure provides microparticle carbon covalently linked to peroxidase. Preferably, the microparticle carbon is native

microparticle carbon conjugated to microperoxidase via a bifunctional reagent.

5 The present disclosure also provides microparticle carbon:peroxidase conjugates useful as an electrode for the determination of an analyte in a sample. The preferred electrode comprises native microparticle carbon covalently linked to microperoxidase in a resin matrix.

Detailed Description of the Invention

10 Embodiments of the invention generally comprise an electrode used to electrochemically measure the concentration of an analyte of interest in a solution. The biosensor or electrode includes a peroxidase enzyme covalently bound to microparticle carbon and retained in a matrix in intimate contact with the electrode.

15 As used herein, the term "microparticle carbon" refers to fine particles of carbon, carbon catalysts (e.g., platinized carbon, ruthenium containing carbon, etc.) and carbon-containing conductive compounds (e.g., polypyrrole, polyaniline). Microparticle carbon in a variety of forms are known in the art and may be readily obtained from commercial
20 vendors. For ease of use and handling, it is preferred that the microparticle carbon be less than 1000 nanometers (1 micron) in diameter, preferably in the range of 5-50 nm. In the experiments described below, a fine (30 nm) particle carbon (Vulcan XC72R; E-TEK, Natick MA) is used.

25 The microparticle carbon can be used in its native state ("native") or various functional groups can be attached to the carbon ("derivatized") following techniques known in the art. For derivatized carbon, the carbon microparticle is reacted with a bifunctional reagent, preferably with a bifunctional agent having amine-containing function groups. As
30 bifunctional reagents are available having a wide variety linkers of differing lengths between the two functional ends, one can easily and rapidly determine those reagents which are suitable for use with a variety of enzymes and for a variety of applications. The resulting derivatized carbon particles can be immediately coupled to the enzyme or
35 can be stored prior to enzyme coupling.

The derivatized or native particles are covalently attached to the enzyme, again following techniques known in the art, and as described in more detail below.

5 Peroxidase of any origin is useful in the interference free biosensor of the invention. Peroxidase is readily obtained following techniques known in the art, or alternatively, may be purchased from commercial vendors. In a preferred embodiment, the peroxidase is microperoxidase such as described European Patent Publication 0 195 320.

10 As microparticle carbon with attached enzyme cannot by itself be readily used for the detection of an analyte of interest, the carbon should be physically confined in such a manner that the analyte may be brought into contact with the carbon:enzyme conjugate. Any of the confinement means known in the art of e.g., gold:peroxidase conjugates, are suitable for use in the present invention as well. For example, by the use of semi- or
15 selectively-permeable membranes, the carbon can be physically restrained while allowing the analyte of interest to come into intimate contact with the peroxidase enzyme. In a preferred embodiment, the microparticle carbon:enzyme conjugate is used for construction of an electrode. In this particular embodiment, the microparticle
20 carbon:enzyme conjugate is confined to an electrically conductive surface in order to form an electrode. Of the many ways of confining the carbon:enzyme microparticle to a surface which are known in the art, the procedure described in International Patent Publication WO 95/22057 is preferred. The method described therein incorporates the particles into a
25 resin matrix (also referred to as "paint") and has the benefits of being water based to preserve enzyme activity. It also yields a liquid, facilitating dispensing and easing the manufacturing of electrodes. As disclosed therein, the liquid resin with the contained carbon particles dries to form a water insoluble film which is compatible with blood.

30 The enzyme:microparticle carbon of the invention is useful in any application where the catalytic properties of peroxidase prove beneficial. Significantly, the enzyme:microparticle carbon of the invention provides a composition which is essentially free of interference from compounds which normally interfere with peroxide determinations. Thus the
35 enzyme:microparticle carbon of the invention can be used in instrument

based systems (in vitro) or in devices for the direct measurement of blood analytes in vivo.

Example 1 – Covalent coupling of microperoxidase to carbon particles

5 In this experiment, native and two forms of derivatized carbon particles are covalently coupled to microperoxidase.

10 The first form of derivatized carbon utilizes the functional group ethylenediamine (Aldrich, Milwaukee WI) attached to carbon by mixing 100 mg of carbon with 27 mg of ethylenediamine in 5 mL of a 50 mM 2[N-Morpholino]-ethanesulfonic Acid (MES) (Sigma, St. Louis MO) buffer at pH 5.5. 50 mg of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Pierce, Rockford IL) is then added to initiate the coupling and allowed to react for 1 hour. The derivatized carbon is removed by centrifugation and washed three times with water.

15 In a second form of derivatized carbon, a poly(oxyalkylene)diamine (JEFFAMINE® ED-600; Texaco, Houston TX) is employed as the functional reagent and is attached to carbon by mixing 100 mg of carbon with 240 mg of the diamine in 5 mL of a 4% pyridine/water solution at pH 5.5. 4 mg of EDC is then added to initiate the coupling and allowed to react overnight. The derivatized carbon is then removed by centrifugation and washed three times with water.

20 The peroxidase enzyme is covalently coupled to the native or derivatized microparticle carbon using EDC. 100 mg of carbon is mixed with 20 mg of the peroxidase enzyme (for example, MP-11 microperoxidase from Sigma Chemical, St. Louis, MO) in 5 mL of a 50 mM MES buffer, pH 5.5. Alternatively, and preferably, the buffer can be 4% pyridine/water solution at pH 5.5. 10 mg of EDC is added and the solution is mixed for 1 hour. The carbon with covalently attached peroxidase enzyme is removed by centrifugation and washed with water. The carbon with attached enzyme is stored wet.

25 To provide a comparison to covalent coupling, enzyme adsorption is done in a solution of 50 mM sodium phosphate, 1 mM EDTA, 100 mM sodium chloride, and 0.05% antimicrobial (Kathon CG; Rohm and Haas, Philadelphia PA) solution at a pH of 7.5. This solution is referred to as PESK 7.5. 125 mg of carbon is mixed with 25 mg of the enzyme in 5 mL

30

35

- 6 -

of PESK 7.5 overnight. The carbon is then removed by centrifugation and washed three times with PESK 7.5.

Example 2 – Preparation of the Paint

- 5 A resin matrix for retaining the carbon on a conductive surface, termed F133 Resin, is made by combining the following weights of each component:

Joncryn 537 (Johnson Wax, Racine WI)	65.04 mg
NH ₄ OH, 10% in water	8.91 mg
2-Ethoxy-ethanol (Aldrich, Milwaukee WI)	20.94 mg
Dibutyl Phthalate (Fluka, Ronkonkoma, NY)	7.13 mg
Surfynol 104H (Air Products, Allentown, PA)	2.50 mg
Surfynol 695 (Air Products)	5.0 mg
Acrysol 275 (Rohm and Haas, Philadelphia, PA)	30.6 mg

- 10 72 mg of the resin, a liquid, is then combined with 160 mg of the carbon:enzyme conjugate compacted in the wet state, 262 mg of 0.085 M trehalose (Sigma, St. Louis, MO) in water. A vigorous mixing of the three components is done using an amalgamator (WIG-L-BUG; Crescent Dental Co., Lyons IL) for 3 minutes, forming a liquid suspension. This suspension
15 or paint may then be applied to an electrode.

Example 3 – Electrode construction

The carbon:enzyme conjugates are utilized in an analytical electrode of the type described in International Patent Publication WO 95/22057.

- 20 Briefly, an analytical electrode assembly for testing the carbon:enzyme conjugates of the invention is shown in Figure 1. Electrode assembly 10 consists of one or more (in this figure, four) biosensors 12 mounted on a polyester sheet 32. The biosensors 12 are constructed by printing silver/silver chloride (Acheson SS24540) electrical connections
25 20 on the polyester sheet 32. At the location for electrical contact with the instrument 21 as well as the terminus of the electrical connection 20, an electrically conducting trace is formed by screen printing of carbon ink (Acheson 423SS) over the electrical connection 20. The carbon surface is

masked by the application of a screen printed dielectric (Acheson ML 25208), leaving 0.013 cm² of carbon exposed for application of the paint at 12. 0.2 to 1.0 µl of carbon:enzyme conjugate paint is applied to the exposed surface of the carbon trace using a hand dispenser. Reference electrode 14 is printed using silver/silver chloride (Acheson SS24540). An additional counter electrode (not visible in Figure 1) is formed on face plate 18 on the side facing gasket 16. The counter electrode is printed with carbon as described above, however no paint is applied. Gasket 16 provides a flow channel 34 when sandwiched between polyester sheet 32 and front plate 18. Fluid may then flow over the biosensors 12, reference electrode 14, and the counter electrode (not shown) from entry port 24 exiting via exit port 26. Back plate 22 may be fastened to front plate 18 via a fastening means such as a nut 28 inserted through fastening hole 42 to bolt 30. Optionally, alignment means may be provided to ensure proper registry of the components via holes 44 and pins 46.

Example 4 – Demonstration of sensitivity

a. Several electrodes are fabricated as described in Examples 1 through 3, utilizing different enzymes (horseradish peroxidase, soybean peroxidase, and microperoxidase), different carbon attachment methods (adsorption or covalent coupling), and different carbon treatments (native, ethylenediamine, or JEFFAMINE ED-600). A test solution of about 0.1 mM hydrogen peroxide at 37 °C is passed over the carbon trace, the reference electrode, and the counter electrode. A fixed electrical potential of 100 mV is applied to the electrode, and the resulting electrical current is recorded for various electrode types using a standard three electrode circuit. The measured currents for various combinations of enzyme, carbon attachment technique, and carbon treatment are as follows:

Enzyme	Carbon Attachment	Carbon Treatment	Current (nA)
Horseradish Peroxidase	Adsorption	None	-34
Horseradish Peroxidase	Covalent	Ethylene-diamine	-187
Horseradish Peroxidase	Covalent	JEFFAMINE	-324

- 8 -

Soybean Peroxidase	Covalent	Ethylene-diamine	-407
Soybean Peroxidase	Covalent	JEFFAMINE	-442
Microperoxidase	Adsorption	None	-46
Microperoxidase	Covalent	None	-1023

As apparent from the foregoing, covalently attached microperoxidase shows much larger currents than the conventional method of adsorption. Covalently attached microperoxidase (MP-11, Sigma, St. Louis MO) yields much larger currents than other peroxidase enzymes such as horseradish peroxidase (Type II, Sigma) and soybean peroxidase (Enzymol International, Inc., Bexley OH).

- b. Potassium ferricyanide also reduces in a reversible reaction that is similar to peroxidase. It is well recognized in the art, see, e.g., Csoregi, E., et al, that when equal concentrations of peroxide and ferricyanide are employed, the current produced from the peroxidase is 30-60 X less than the current produced from the ferricyanide. To assess the sensitivity of the present invention, the current resulting from the reduction of peroxide at the present electrodes is compared to the current resulting from the reduction of potassium ferricyanide. Hydrogen peroxide and ferricyanide at substantially equal concentrations of about 0.05 mM at about 37 °C are passed over an embodiment of the electrode described here having microperoxidase covalently coupled to native carbon. Surprisingly, the current for peroxide reduction was twice that as for ferricyanide reduction (see following table).

Analyte	Applied Potential (mV)	Current (nA)
Hydrogen Peroxide	100	-147
Ferricyanide	0	-70

- Because the peroxide reduction involves two electrons as opposed to one for ferricyanide, a signal produced by the present invention which is twice that produced by ferricyanide indicates extremely fast electron transfer from the carbon to the microperoxidase enzyme (at least as fast

as that for the reversible Ferri/Ferrocyanide reaction). This has never been achieved before, and demonstrates the sensitivity of the invention.

Example 5 - Demonstration of Linearity

- 5 Electrodes are made with soybean and horseradish peroxidase covalently coupled to ethylenediamine derivatized carbon and microperoxidase covalently coupled to native carbon. The response of the electrodes to varying levels of hydrogen peroxide at about 37 °C and an electrode potential of 100 mV is measured, with the following results.

10

Enzyme	Current (nA) 0.005 mM H ₂ O ₂	Current (nA) 0.010 mM H ₂ O ₂	Current (nA) 0.100 mM H ₂ O ₂	Current (nA) 0.300 mM H ₂ O ₂
Soybean Peroxidase	-70	-114	-407	---
Horseradish Peroxidase	-69	-102	-257	---
Microperox- idase	-13	-27	-237	-669

- As can be seen, neither soybean nor horseradish peroxidase are linear over the range tested. Readings at higher concentrations are not taken as the response is significantly diminished and thus the information is not informative. On the other hand, microperoxidase demonstrates linearity to at least 0.3 mM hydrogen peroxide.

- In the literature to date, horseradish or fungal peroxidase have been favored over microperoxidase for use in the detection of peroxide. However, embodiments of this invention utilizing microperoxidase far exceed applications with other peroxidase enzymes in not only signal size but also in linearity.

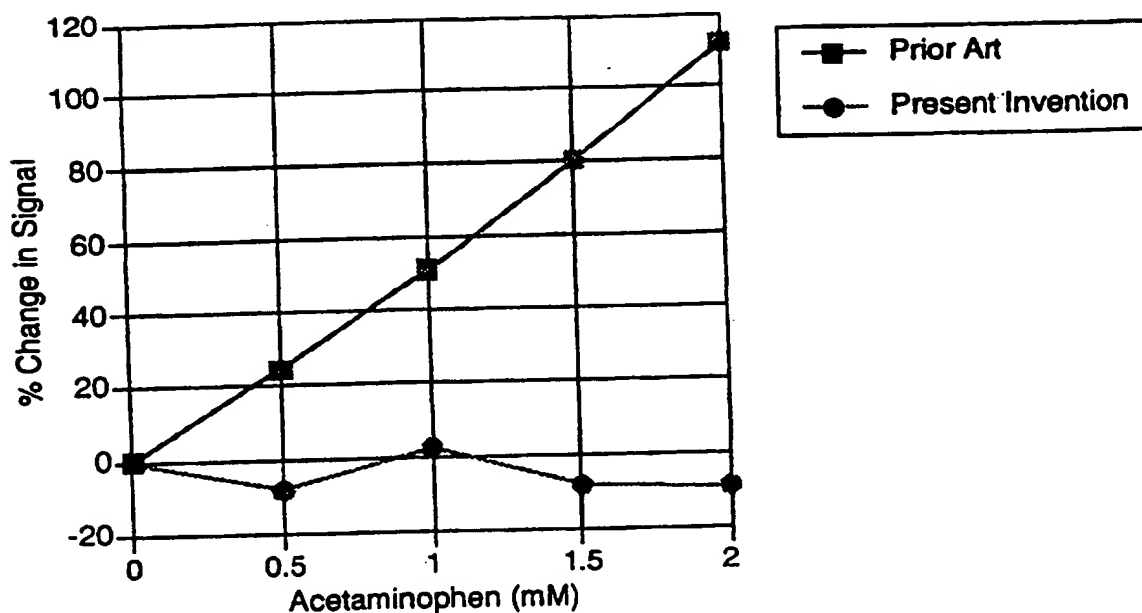
Example 6 - Measurement of glucose concentrations

- 25 In this example, glucose is measured using a commercial instrument (Nova Biomedical Stat Profile 10) in which the paint of the present invention is used in commercial electrodes supplied by the manufacturer.

- 10 -

Microperoxidase is covalently attached to native carbon as described in Examples 1 and a paint is made as described in Example 2. The paint is applied to the center of the interior surface of a Nova Biomedical glucose enzyme cap in such a way that the paint is aligned
5 with the platinum working electrode of the electrode body. The paint is allowed to dry and the cap is then placed on the glucose electrode body. The control electrode is an unmodified glucose electrode, i.e., as supplied by the manufacturer, and is operated in accordance with the manufacturer's instructions. The modified electrode is placed in a flow
10 cell and glucose solutions having varying levels of interfering substances are passed over the electrode, with flow stopped for measurements. The current is measured using a potentiostat, with about 100 mV potential applied. The glucose level is kept constant at about 2 mM at a temperature of about 37 °C to simulate a hypoglycemic diabetic sample.
15 Acetaminophen is varied up to the toxic limit of 2 mM. The percentage change in signal of the electrode in the absence and presence of acetaminophen is quantified, with the results shown below.

%Change in Glucose Signal vs Acetaminophen Concentration. 2 mM Glucose solution.

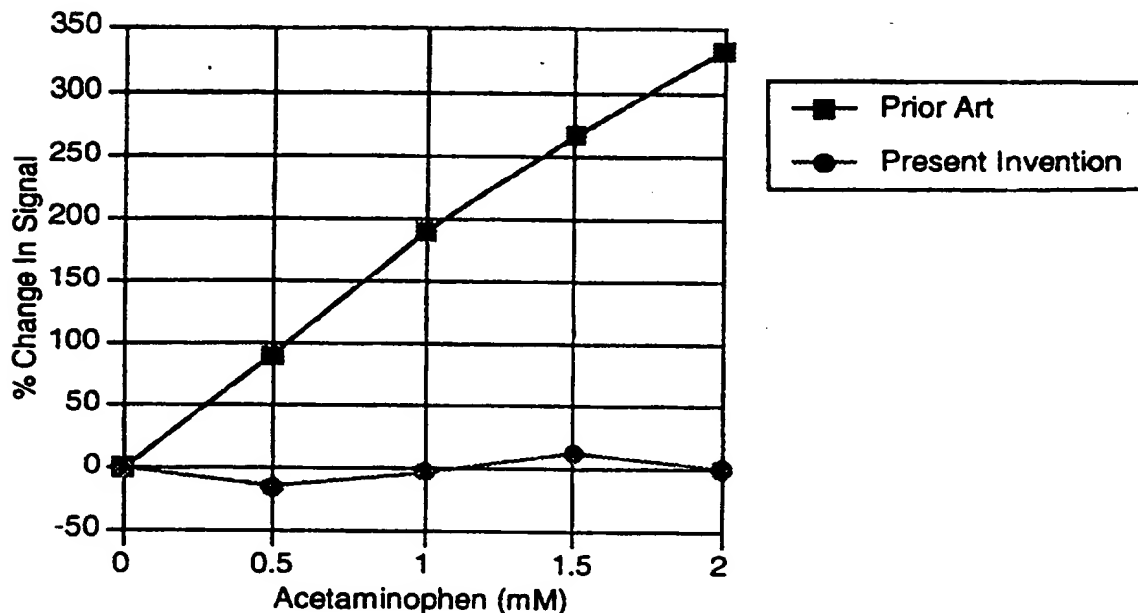


The results indicate that there is essentially no effect of acetaminophen on the glucose measurement of the electrode embodiment described here.

Example 7 – Measurement of lactate concentration

An experiment similar to that of Example 6 is conducted using a Nova Biomedical Lactate enzyme cap. As lactate is found in lower concentrations in blood than glucose, a 0.3 mM lactate solution is used. Again, as shown below, the utilization of the microperoxidase of the present invention eliminates interference due to acetaminophen on lactate measurement.

% Change in Lactate Signal vs Acetaminophen Concentration. 0.3 mM Lactate Solution.



5 Example 8 - Comparison of Microperoxidase to Porphyrins

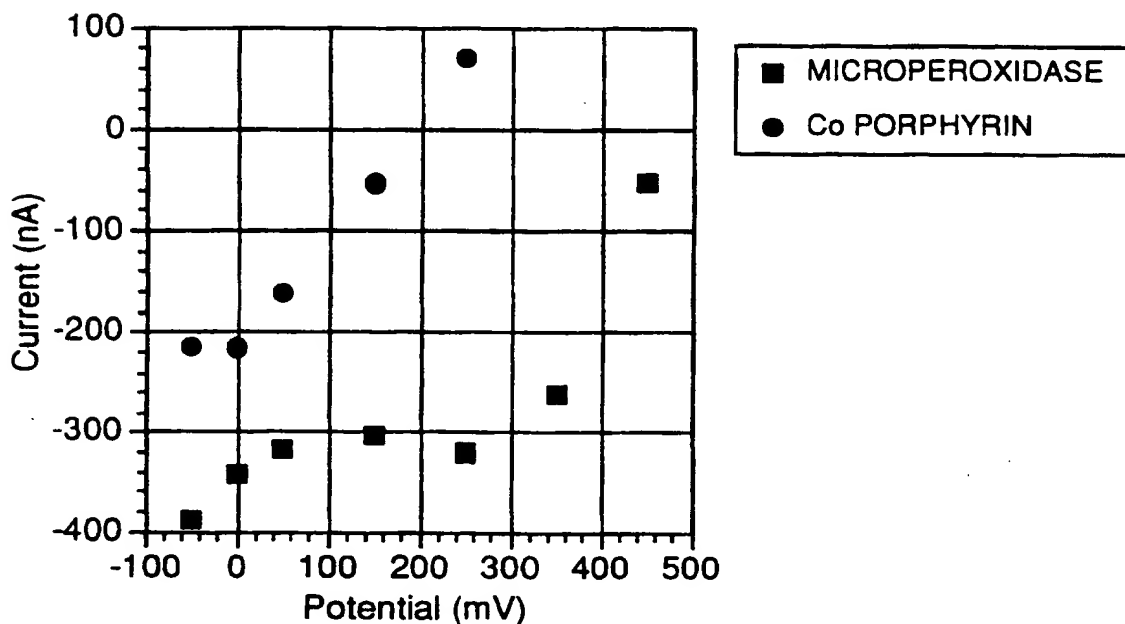
It is known in the art that porphyrin can act as a catalyst. Because microperoxidase is essentially a short peptide chain of 11 amino acids which is covalently attached to a porphyrin ring, and thus many similarities exist in chemical structure between porphyrins and microperoxidase, the following experiment assesses the necessity of the peptide chain in the catalytic reduction of peroxide.

Cobalt is inserted into Protoporphyrin IX dimethylester (Sigma) with the following procedure. 50mg of porphyrin is dissolved in 25mL of DMF in a 100mL round bottomed flask. 50 mg of the cobalt chloride (Sigma) is added and refluxed until the florescence is quenched. The solution is then cooled and cold water is added to recrystallize the cobalt porphyrin. After washing and drying, cobalt porphyrin is adsorbed to 250 mg of wet carbon. The adsorption is done by mixing 10 mg of dry porphyrin with carbon in 5 mL of chloroform. After removing chloroform

- 13 -

- by washing with acetone and water, the cobalt porphyrin is made into a paint by mixing about 160 mg of carbon:adsorbed porphyrin, 72 mg of F133 resin, and 262 mg of 0.085 M trehalose in water. The paint is dispensed on the screen printed electrode described in Example 3. The reduction of 0.1 mM hydrogen peroxide is recorded for cobalt porphyrin electrodes and microperoxidase (covalently linked to native carbon) electrodes at different potentials.

REDUCTION OF 0.1 mM HYDROGEN
PEROXIDE AS A FUNCTION OF POTENTIAL



10

- These results show that a microperoxidase containing paint of the present invention is capable of reducing peroxide at potentials approximately 400 mV more easily than cobalt porphyrin. This indicates that microperoxidase performs significantly better than porphyrin in the catalytic reduction of peroxide.

15

Example 9 - Synthesis of Alternate Microperoxidase Enzymes

- 14 -

The following experiment is designed to explore the necessity of iron to the catalytic property of microperoxidase. The results indicate that the metal moiety is necessary for catalytic activity, and, surprisingly, activity is retained when the native iron is removed and replaced with other metals.

a. Iron is removed from microperoxidase by adding 800 mg of iron sulfate in 4mL of concentrated HCl to 2000 mg of enzyme dissolved in 14mL of glacial acetic acid. This reaction mixture is left stirring under nitrogen for 10 minutes during which the maroon color of the solution turned purple. Acetone is added to the solution until the microperoxidase precipitates. This solid is then collected, washed by centrifugation with acetone, and dried.

b. To insert the various metals, 20 mg of the microperoxidase from Example 9a (referred to hereafter as "deironated" peroxidase) is dissolved in 4 mL of a 1:3 pyridine:acetic acid solution. The solution is heated to 80 °C under nitrogen and 20 mg of metal (e.g., iron, zinc, copper, nickel) in the form of the sulfate or chloride salt, is added in 1mL of water. The mixture is reacted under nitrogen at 80 °C for 10min. followed by cooling in air for 10min. Acetone is added to precipitate the microperoxidase, and the solids are washed with acetone.

Each microperoxidase containing a different metal is tested for activity prior to covalent coupling to carbon. The activity is measured by observing the rate of formation of the quinoneimine dye from 4-aminoantipyrine and 2-hydroxy-3,5-dichlorobenzene sulfonic acid at 550 nm in the presence of an excess of hydrogen peroxide. The following relative activity results are determined.

- 15 -

Micro- peroxidase	Activity ($\mu\text{g/mL}$)
Untreated	3.79
"De-ironated"	0.008
Iron	2.848
Zinc	1.694
Copper	0.128
Nickel	1.78

The results indicate an approximately 25% loss in activity following removal and replacement of iron. The zinc and nickel microperoxidases show approximately a 50% loss in activity. Copper microperoxidase is not very active.

The microperoxidase enzymes containing different metal ions are covalently coupled to native carbon using the procedure described in Example 1. Paints are prepared using about 160 mg of carbon:enzyme conjugate, 72 mg of F133 resin, and 262 mg of 0.085 M trehalose in water, after which it is dispensed on carbon traces, as described in Example 3, for testing. The electrodes are tested for the reduction of hydrogen peroxide at 100 mV.

Micro- peroxidase	Current (nA)
Iron (untreated)	-800
Nickel	-560
Zinc	-190
Copper	-20

These results show that microperoxidase can be modified by insertion of a different metal ion and used in a sensor to detect peroxide.

All of the references cited in this application are incorporated by reference.

The present invention has been described with reference to preferred embodiments. One of skill in the art will readily appreciate that changes, alterations or modifications can be made to these
5 embodiments without departing from the true scope and spirit of the invention.

CLAIMS

We claim:

1. Microparticle carbon covalently linked to peroxidase.
2. The composition of Claim 1 wherein the microparticle carbon is native microparticle carbon.
3. The composition of Claim 1 wherein the microparticle carbon is derivatized with a bifunctional reagent.
4. The composition of Claim 3 wherein the bifunctional reagent is a diamine.
5. The composition of Claim 4 wherein the diamine is a poly(oxyalkylene) diamine.
6. The composition of Claim 1 wherein the microparticle carbon is carbon or a carbon catalyst.
7. The composition of Claim 1 wherein the microparticle carbon is a carbon-containing conductive compound.
8. The composition of Claim 6 wherein the microparticle carbon is carbon and the peroxidase is microperoxidase.
9. The composition of Claim 8 wherein the microparticle carbon is native microparticle carbon.
10. The composition of Claim 1 wherein the metal moiety of the peroxidase is modified to contain iron, nickel or zinc.
11. The composition of Claim 1 further comprising a resin matrix.

- 18 -

12. An electrode for the determination of an analyte in a sample comprising:

microparticle carbon conjugated to peroxidase confined to an electrically conductive surface.

13. The electrode of Claim 12 wherein the microparticle carbon is native microparticle carbon.

14. The electrode of Claim 13 wherein the peroxidase is microperoxidase.

15. The electrode of Claim 14 wherein the microparticle carbon conjugated to peroxidase is confined in a resin matrix.

16. A method of measuring an analyte of interest in a test sample, comprising contacting the sample with microparticle carbon conjugated to peroxidase, and detecting the change in electrical potential as a quantitative or qualitative measure of the amount of analyte in the sample.

17. The method of Claim 16 wherein the microparticle carbon is native carbon.

18. The method of Claim 17 wherein the peroxidase is microperoxidase.

19. The method of Claim 16 wherein the microparticle carbon conjugated to peroxidase is confined to an electrically conductive surface.

20. The method of Claim 19 wherein microparticle carbon conjugated to peroxidase is confined in a resin matrix.

21. The method of Claim 16 wherein the analyte is glucose or lactose.

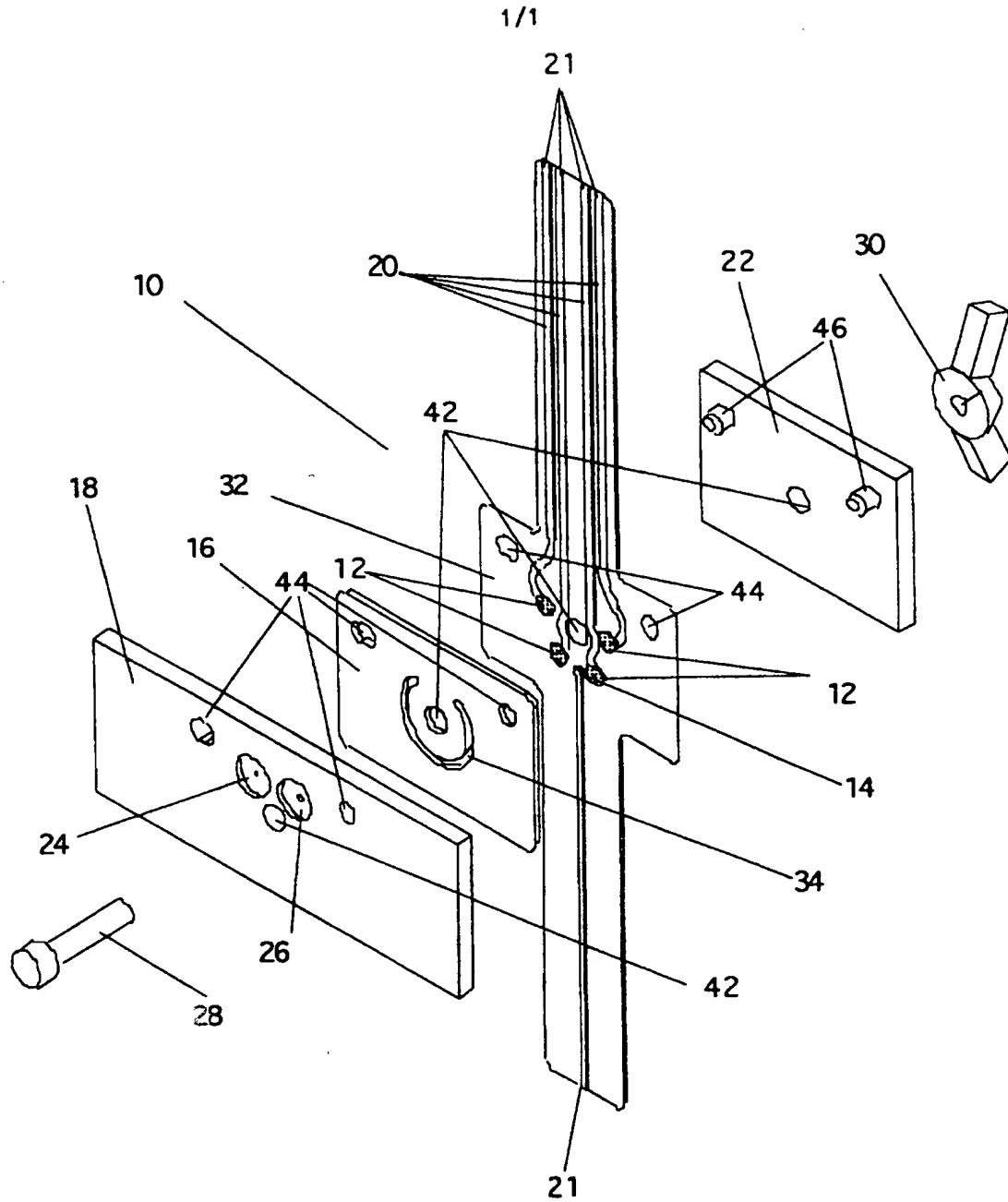


Figure 1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/18889

A. CLASSIFICATION OF SUBJECT MATTER C 12 Q 1/28, C 12 Q 1/54, G 01 N 27/327		
According to International Patent Classification (IPC) or to both national classification and IPC ⁶		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C 12 Q, G 01 N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 119, no. 4, issued 1993, July 26, (Columbus, Ohio, USA), E. CSOEREGI et al. "Carbon fibers as electrode materials for the construction of peroxidase-modified amperometric biosensors", page 859, no. 39 728u; & Anal. Chim. Acta 1993, 273(1-2), 59-70.	1-21
A	CHEMICAL ABSTRACTS, vol. 102, no. 22, issued 1985, June 3, (Columbus, Ohio, USA), J. JASAITIS et al. "Acceler- ation of electrode processes by biocatalysts. 10. Macro- kinetic characteristics of	1-21
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*Z* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search 13 February 1997		Date of mailing of the international search report 05-03-1997
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer SCHNASS e.h.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/18889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	hydrogen peroxide reduction on rotating peroxidase graphite electrodes", page 568, no. 193 765j; & Liet. TSR Mokslu Akad. Darb., Ser. B 1984, (6), 34-40. --	
A	CHEMICAL ABSTRACTS, vol. 116, no. 8, issued 1992, February 24, (Columbus, Ohio, USA), G. JOENSSON-PETTERSSON "Re- agentless hydrogen peroxide and glucose sensors based on peroxidase immobilized on graphite electrode", page 1093, no. 75 321 c; & Electroanalysis (N.Y.) 1991, 3(8), 741-50. --	1-21
A -	CHEMICAL ABSTRACTS, vol. 119, no. 17, issued 1993, October 25, (Columbus, Ohio, USA), J. MARCINKEVICIENE et al. "Biosensor strip-type glucose sensor", page 388, no. 176 849y; & Biosens. Bioelectron. 1993, 8(3-4), 209-12. --	1-21
A	CHEMICAL ABSTRACTS, vol. 119, no. 16, issued 1993, October 18 (Columbus, Ohio, USA), J. WANG et al. "In situ eli- mination of metal inhibitory effects using ligand-contain- ing carbon paste enzyme electrodes", page 926, no. 173 169r; & Anal. Chem. 1993, 65(19), 2698-700. ----	1-21